Sequence elements surrounding the acceptor site suppress alternative splicing of the sarco/endoplasmic reticulum Ca^{2+}-ATPase 2 gene transcript

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Expression of the muscle-specific 2a isoform of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA2) requires activation of an inefficient optional splice process at the 3′ end of the primary gene transcript. The sequence elements required for this regulated splice event were studied by modifying a minigene containing the 3′ end of the SERCA2 gene. An important requirement appears to be a strong muscle-specific acceptor site, as replacing it by a weak one prevented the induction of muscle-type splicing during myogenic differentiation. The induction of muscle-type splicing did not depend on positive *cis*-active sequences in the muscle-specific exon. On the other hand, replacement of a broad region around the acceptor site dramatically deregulated the expression pattern, as this modification strongly induced muscle-type splicing in undifferentiated muscle cells and in fibroblasts. This *cis*-active region is also involved in the suppression of the neuronal type of splicing. Furthermore selective replacement of the acceptor site as well as deletions or replacements in the muscle-specific exon induced muscle-type splicing to various extents in undifferentiated myogenic cells. Therefore sequence elements in the distal part of the optional intron and in the muscle-specific exon contribute to the suppression of muscle-specific SERCA2 splicing.

INTRODUCTION

Three different genes encode the Ca^{2+}-transport ATPase present in the sarcoplasmic or endoplasmic reticulum (SERCA). Additional alternative processing of the primary messenger leads to five different SERCA isoforms mediating the uptake of Ca^{2+} into intracellular stores (for a review see [1]). These different SERCAs are expressed in a tissue-specific and developmentally regulated way. For SERCA2, tissue-specific alternative processing of the 3′ end generates two protein isoforms. These isoforms differ in their C-terminal region and are functionally different. The muscle-specific SERCA2a has a lower Ca^{2+} affinity and a higher turnover rate for Ca^{2+} transport and ATP hydrolysis than the non-muscle SERCA2b [2,3].

At the mRNA level, four different SERCA2 mRNAs (class 1 to 4) are formed (Figure 1). The class-1 messenger is generated by removing an optional intron at the 3′ end of the SERCA2 transcript. This messenger is found in cardiac muscle, slow-twitch skeletal muscle and smooth muscle and is translated into the SERCA2a isoform. The class-2 mRNA is polyadenylated at a site within the optional intron and is found in non-muscle cells. The class-3 mRNA is polyadenylated at a downstream site and it contains the optional intron. This messenger is present at a low concentration in all tissues. The class-4 messenger is formed by a splicing process using a second donor more proximal to the same acceptor site than used in class 1. The class-4 messenger is restricted to the brain and, like class 2 and class 3, encodes the non-muscle SERCA2b isoform.

The alternative processing of the SERCA2 gene transcript was studied using a minigene containing the 3′ end of the SERCA2 gene. SERCA2 transcript processing was studied by stably transfecting the minigene in a myogenic cell line (BC_3H1) well characterized with respect to differentiation-dependent SERCA2 processing [4,5]. Previous studies on the regulation of alternative 3′-end processing have shown the following. (1) The muscle-specific splicing and not the mutually exclusive internal polyadenylation is the regulated process [6]. (2) Different structural mRNA elements are necessary for regulated splicing to occur (Figure 1). These include weak 5′ splice sites, a weak internal polyadenylation site and the presence of a long terminal intron [7]. (3) This structural organization is not sufficient for splice regulation. Muscle-specific *trans*-acting factors are necessary to obtain splicing [8]. In this study, we wanted to identify further

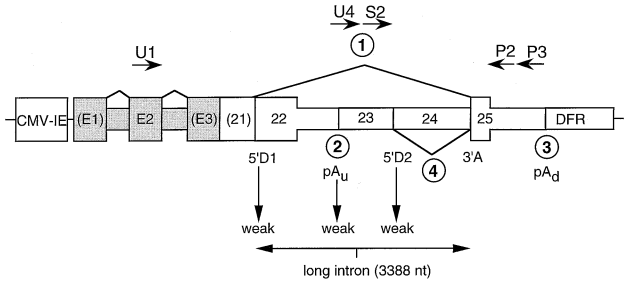

Figure 1 Schematic representation of the β-globin and SERCA2 region in pCMβSERCA2

Part of the pCMβSERCA2 minigene is schematically shown as well as the already known prerequisites for regulated splicing. The processing signals present in SERCA2 are indicated as follows: 5′D1 and 5′D2, 5′ donor splice sites 1 and 2 respectively; 3′A, 3′ acceptor splice site; pA_u and pA_d, upstream (u) and downstream (d) polyadenylation signal. The β-globin regions are shaded, and the SERCA2 sequences are open boxes. Partial exon sequences are in parentheses, and coding and non-coding sequences are represented by wide and narrow boxes respectively. The numbers refer to the alternative processing patterns giving rise to the different mRNA classes. Depicted as arrows above the sequence are the forward and reverse PCR primers used to amplify the different RNA classes. CMV-IE, cytomegalovirus-immediate early promoter; DFR, downstream flanking region.

Abbreviations used: SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; RT, reverse transcriptase.

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the sequence elements on which these unknown *trans*-acting factors act.

Different modes of regulation can be envisaged. One possibility is that regulation is achieved via splice enhancers. During recent years, two types of such splice enhancers have been identified in several models of alternative splicing: (a) intronic elements characterized as short repeats involved in the cell-type-specific inclusion of the upstream optional exon (e.g. exon IIIB of the fibronectin gene [9,10]); (b) exon elements identified as purine-rich regions, which are believed to act as binding sites for SR-splice factors [9,10,11–14]. In a previous study we found no evidence that SR proteins are critical for the regulation of SERCA2 splicing [8].

Tissue-specific splice regulation can also be obtained via negative *cis*-active elements, which interact with a *trans*-acting splice suppressor. These suppressors often block splicing by binding to sequences surrounding alternatively used splice sites. In this way they preclude the use of these sites in spliceosome formation. This has been described for the 3′ splice acceptor site (e.g. alternative splicing of transformer in *Drosophila* [15–18]) as well as for sequences close to the 5′ donor splice site (e.g. alternative splicing of the P-somatic inhibitor [19–21]). Intronic inhibitory elements located centrally in an alternatively spliced intron have also been described (e.g. alternative splicing of the sex-lethal exon 3 in *Drosophila* [22,23]).

In this study we screened for the presence of specific *cis*-active sequences, either splice enhancers or splice suppressors, involved in tissue-specific regulation of SERCA2 splicing. We present evidence for the presence of sequence elements surrounding the acceptor site that inhibit both muscle-specific and the neuronal-specific splicing.

MATERIALS AND METHODS

Minigene modifications

The construction of the recombinant pCMβSERCA2 minigene containing the SERCA2 3′ end has been described previously [6], and the relevant part of this construct is schematically shown in Figure 1. For this study, different new modifications of the wild-type minigene were made.

In the optional muscle-specific exon 25, different deletions were made. Exon deletion (ED) 2 was created starting from Mut12 in which a unique *Asp*718 site is present just upstream of the downstream polyadenylation site (pA_d). For construction of ED2, a *Mun*I–*Asp*718 fragment (387 nt) was removed from the exon and, after blunt-ending, the plasmid was recircularized. The deletion in ED3 was created starting from Mut11. This modified construct contains an *Asp*718 site at the beginning of exon 25. An *Asp*718–*Mun*I fragment was removed from the construct and was replaced by a PCR fragment. This PCR fragment was amplified using Mut11 plasmid DNA (1 ng) as template. Forward primer Fw1 (position 5526–5537; 5′-CAATACTGGGGTACC-CGCTTCC-3′) and reverse primer Rev1 (position 5609–5628; 5′-TGCTCAATTGATGCAGAGGGCTGGTAGATG-3′) were used (the numbering refers to the genomic sequence [24]). The Fw1 and Rev1 primers contain an *Asp*718 and *Mun*I restriction site respectively. After amplification, the PCR fragment was digested with *Asp*718–*Mun*I and ligated into the minigene lacking the *Asp*718–*Mun*I fragment. This resulted in a deletion of 288 nt.

ED1 was created by using a forward primer that is the inverse complement of Rev1 primer with an *Asp*718 site added to its 5′ end (Fw2: 5′-TGCTGGTACCCATCTACCAGCCCTCTGC-AT-3′). The reverse primer used was Rev2 (position 5909–5932 5′-GGCATGCATTACAATTGAATATATTG-3′). After digestion of the amplified fragment with *Asp*718–*Mun*I, it was ligated into the minigene lacking the *Asp*718–*Mun*I fragment. This resulted in a deletion of 72 nt.

The muscle-specific exon was removed from the minigene and was replaced by different unrelated sequences. For exon swap (ES) 1 the *Asp*718–*Eco*RI fragment was removed from Mut11 and was replaced by the *Eco*RI–*Nde*I fragment from the rabbit β-globin gene. As in this mutant also the SERCA2 downstream flanking region was removed from the construct and since this could further affect the transcript processing, we created new substitutions in which exon 25 sequences were selectively removed. Therefore Mut14 containing an *Asp*718 restriction site at the beginning and end of exon 25 was used. The *Asp*718–*Asp*718 fragment was replaced by a λ-phage DNA fragment. A 1924 nt-long PCR fragment amplified using λ-phage DNA (molecular mass marker III; Boehringer-Mannheim, Mannheim, Germany) was used as a template. This PCR fragment was cut with *Bst*EII–*Stu*I resulting in a fragment of 912 nt. This fragment was ligated into the construct lacking the *Asp*718–*Asp*718 fragment, resulting in ES2.

For acceptor swaps (AS) 1 and 2, a second *Asp*718 restriction site was created 60 nt upstream of the *Asp*718 site in exon 25. In AS1, a *Bsp*120I–*Eco*RI fragment from the rabbit β-globin gene containing the acceptor region of exon 3 was used to replace the SERCA acceptor, and in AS2, a *Mun*I–*Apa*L1 fragment of 82 nt containing the acceptor region of the constitutive Cμ3 exon from Igμ was used. In AS3 and AS4, the acceptor region of the muscle-specific exon was removed by eliminating a *Xho*I–*Asp*718 fragment. A *Hin*cII–*Bgl*II fragment (AS3) and a *Hin*cII–*Eco*RI fragment (AS4) from the rabbit β-globin gene containing the acceptor region of exon 3 were used to replace the SERCA acceptor region.

Cell culture, DNA transfections, RNA isolation and reverse transcriptase (RT)-PCR

BC_3H1 mouse myoblasts (ATCC; CRL1443) were cultured and stably transfected using the calcium phosphate co-precipitation method as described previously [7]. Briefly, 24 h before transfection exponentially growing cells were seeded at a density of 50000/cm². The monolayers were incubated with the calcium phosphate/DNA co-precipitate for 15 min and thereafter growth medium was added. After 48 h, the cells were replated at a lower cell density in selection medium consisting of growth medium containing 400 μg/ml Geneticin (Gibco–BRL, Gaithersburg, MD, U.S.A.). BC_3H1 was induced to differentiate by switching the cells to medium containing 0.5 % (v/v) serum and culturing them for 10 days in this differentiation medium. Differentiation was verified by amplifying the endogenous class-1 messenger of SERCA2. Total RNA was isolated from undifferentiated and differentiated cells by the Chirgwin procedure [25]. RT-PCR (25 cycles; 1 min at 95 °C, 1 min at 55 °C and 1 min at 72 °C) to detect class-1, class-2/3 and class-4 messengers was performed as described by Mertens et al. [7], and the amplification products were separated by electrophoresis on a 6 % polyacrylamide gel. The relative positions of the primers used to amplify fragments of the different SERCA2 transcripts are shown in Figure 1. The muscle-specific class-1 transcript from pCMβSERCA2 was amplified using as forward primer U1 located in exon 2 of β-globin and as reverse primer P2 located in exon 25 of SERCA2. As one primer is located in the β-globin and the other in the SERCA2 part of the construct, pCMβSERCA2 transcripts were specifically amplified. For ED3, a new reverse primer (P3) downstream of the P2 primer was used. It has the following sequence: 5′-CATAGAGAAATCTGGGTAC-3′ (nt 5945–5963,

exon 25). For the exon swaps, two additional reverse primers had to be designed that were located in the sequences replacing the exon 25 sequence. The P4 primer (5′-AGAGGGAAAAAGATCTCAGTG-3′) corresponds to nt 1414–1434 in exon 3 of the rabbit β-globin sequence as described by Van Ooyen et al. [26]. The P5 primer (5′-CAACGACGGCACGAACGGGCAGAT-3′) is located in the λ-phage DNA sequence published by Sanger et al. [27] (position 12971–12994). The neuronal-specific class-4 mRNA was amplified using the U4 sense and P2 antisense primer in the first PCR (25 cycles), and the S2 sense primer and the same antisense primer in the second PCR (10 cycles) [7].

The exact nature of each PCR fragment was determined by sequencing and/or restriction enzyme digests. This was achieved by either cloning or labelling the PCR fragments with [α-^{32}P]dCTP as described previously [5,28]).

RESULTS

The muscle-specific exon 25 contains no splice enhancer, but is involved in splice suppression

To identify potential regulatory *cis*-active sequences in the muscle-specific exon 25, three different deletions were created (Figure 2A). Altogether these deletions span the entire exon. The modified minigenes were transfected in BC_3H1 cells and RNA from undifferentiated and differentiated cells was isolated. The RNA was analysed using RT-PCR. The U1–P2 primer pair was used for the wild-type, ED1 and ED2 minigenes and the U1–P3 primer pair for the ED3 minigene because the binding region for P2 was removed in the latter deletion. Both primer combinations specifically amplify a fragment of the muscle-specific class-1 mRNA. The length of the PCR product varies according to the length of the deletion. For the wild-type and ED2 constructs a fragment of 617 nt is expected, whereas for ED1 and ED3 the expected length is 545 nt and 426 nt respectively. RT-PCR for class-1 mRNA was carried out in the non-saturating range of amplification, and the expression levels of the transfected constructs were all in the same range, as verified by amplification of the class-2/3 fragments (results not shown).

In ED1, 72 nt were removed at the 5′ end of exon 25. As shown in Figure 2(B), splice regulation was conserved with a differentiation-dependent induction of class-1 mRNA (lanes 3 and 4). A very weak signal for the class-1 messenger could be detected in undifferentiated cells (lane 3, Figure 2B). In ED3, 288 nt located downstream of the part removed in ED1 were deleted. The effect of this deletion is shown in Figure 2(C) (lanes 3 and 4). Some class-1 mRNA could already be detected in undifferentiated cells, but splice regulation clearly remained differentiation-dependent. ED2 is a 387 nt deletion located distally to ED3. This deletion removes the second half of the exon. The result of this deletion is shown in Figure 2(D) (lanes 3 and 4). We detected a small amount of spliced mRNA in undifferentiated muscle cells but myogenic up-regulation of splicing was again unaffected.

To study further the potential effect of exon 25 sequences on regulated SERCA2 mRNA processing, two different exon swaps were constructed. In ES1, the entire exon 25 together with the downstream flanking region were removed from the minigene and replaced by part of exon 3 from rabbit β-globin containing the polyadenylation signal and part of its downstream flanking region (Figure 3A). The length of the class-1 fragment amplified by the U1–P4 primer combination is 372 nt. As shown in Figure 3(B), class-1 splicing was already detected in undifferentiated cells and was further up-regulated during myogenic differentiation (lanes 2 and 3).

As the substitution in ES1 affected several elements of the original wild-type minigene that could be important for regulated mRNA processing (exon sequence, polyadenylation site, exon length and downstream flanking region), a second exon swap (ES2) was constructed. All exon 25 sequences were selectively replaced but the polyadenylation signal and the SERCA2 downstream flanking region were retained. For the substitution, a 912 nt λ-phage fragment was used (Figure 3A). The expected

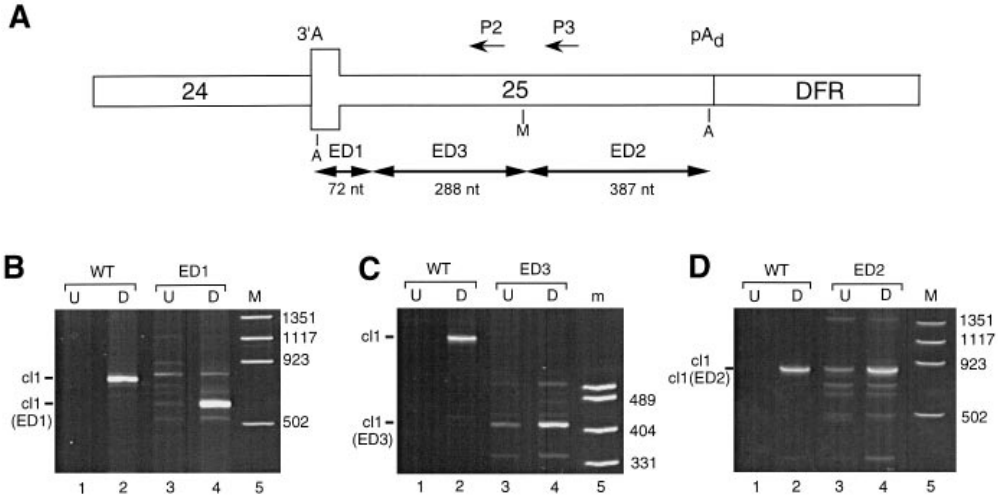

Figure 2 Effect of exon 25 deletions on muscle-specific SERCA2 pre-RNA processing

(**A**) Schematic representation of the deletions made in exon 25. The strategy used for the construction of the different deletions is described in the Materials and methods section. Restriction sites are indicated (M, *Mun*I; A, *Asp*718). The positions of the reverse primers used for RT-PCR amplification of the class-1 fragments are indicated. (**B**), (**C**) and (**D**) The minigenes containing the different exon deletions were stably transfected into BC_3H1 cells, and RNA from undifferentiated (U) and differentiated (D) cells was prepared. For ED1 and ED2 the PCR was performed with the forward primer U1 and the reverse primer P2, and for ED3 the reverse primer P3 was used. cl1 indicates the wild-type (WT) class-1 fragment. cl1(ED1), cl1(ED2) and cl1(ED3) indicate the position of the class-1 fragments corresponding to the respective exon deletions. Lanes M and m contain molecular-size markers with the nucleotide length of the different fragments indicated on the right. 3′A, 3′ acceptor splice site; pA_d, downstream polyadenylation signal; DFR, downstream flanking region.

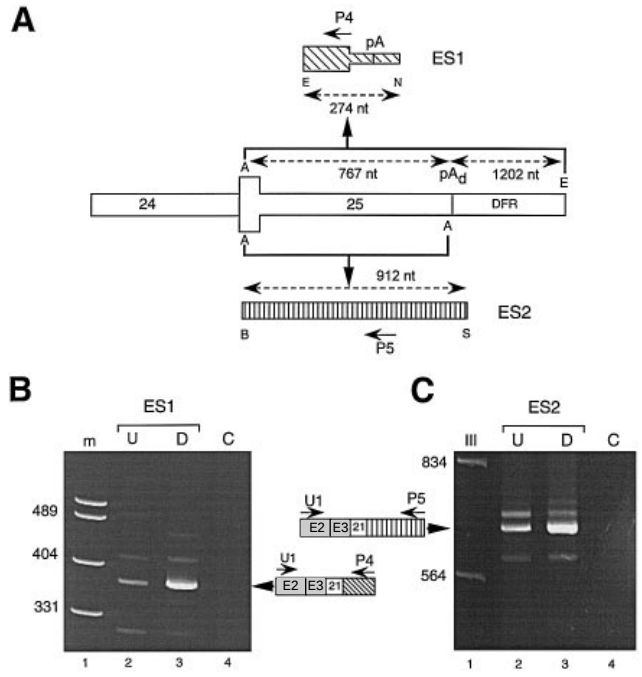

Figure 3 Effect of replacing exon 25 with an unrelated sequence on regulated SERCA2 pre-RNA processing

(**A**) The muscle-specific exon was removed from the minigene and replaced by different unrelated sequences. For ES1 the *Asp*718(A)–*Eco*RI(E) fragment was removed and was replaced by the *Eco*RI–*Nde*I(N) fragment from the rabbit β-globin gene. In ES2 the *Asp*718–*Asp*718 fragment was replaced by a *Bst*EII(B)–*Stu*I(S) λ-phage DNA fragment. (**B**) and (**C**) RT-PCR analysis of alternative transcript processing on RNA obtained from stably transfected undifferentiated (U) and differentiated (D) BC_3H1 cells. For ES1 the U1 primer was used as forward primer with the P4 primer as a reverse. For ES2, the U1 primer was used together with the P5 primers. The amplified fragments and primers are shown next to the gels. In the control lane (C), the cDNA samples were replaced by water, and lanes m and III contain molecular-size markers with the length of the fragments indicated on the left. PA, β-globin polyadenylation signal; pA_d, downstream polyadenylation signal; DFR, downstream flanking region.

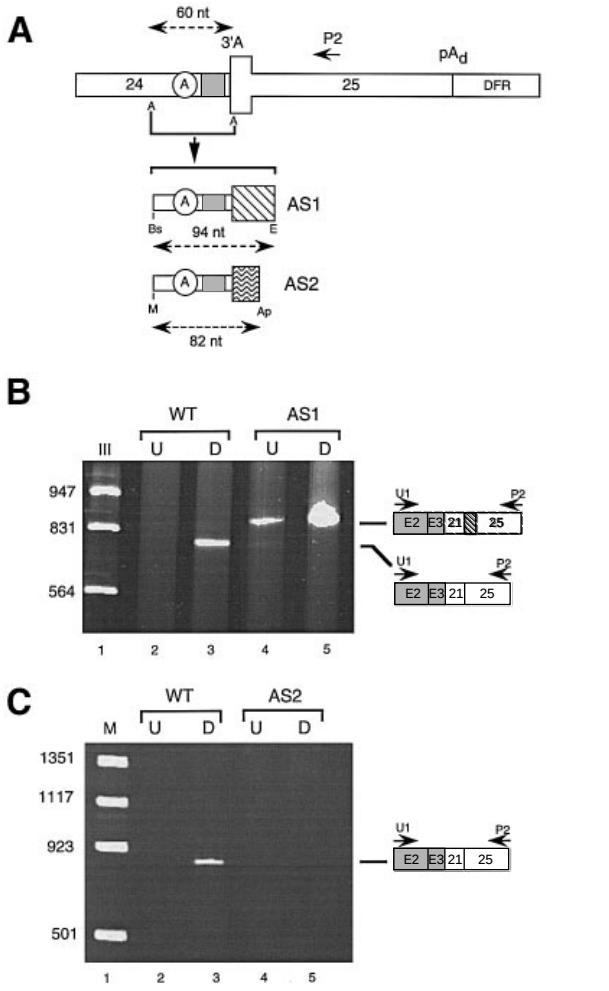

Figure 4 Effect of replacing the acceptor and branchpoint by a β-globin and IgM acceptor on regulated SERCA2 pre-RNA processing

(**A**) Schematic representation of the acceptor swaps 1 (AS1) and 2 (AS2). The encircled A indicates the putative branchpoint adenosine. The shaded boxes represent a polypyrimidine tract. A second *Asp*718(A) restriction site was created 60 nt upstream of the *Asp*718 site in exon 25. The *Asp*718–*Asp*718 fragment containing the SERCA2 acceptor, branchpoint and polypyrimidine tract was replaced by a *Bsp*120I(Bs)–*Eco*RI(E) fragment from the rabbit β-globin gene containing the acceptor region of exon 3 in AS1, and a *Mun*I(M)–*Apa*L1(Ap) fragment containing the acceptor region of the constitutive $C_\mu 3$ exon from IgM was used in construct AS2. (**B**) and (**C**) RT-PCR analysis of alternative transcript processing on RNA obtained from stably transfected undifferentiated (U) and differentiated (D) BC_3H1 cells. The U1 primer was used as forward primer with the P2 primer as a reverse. The amplified fragments and the primers are shown on the right of the gels and lanes III and M contain molecular-size markers.

length of the U1–P5 PCR fragment for class-1 mRNA is 658 nt. As shown in Figure 3(C), a splice pattern identical with that for ES1 was obtained, with a low level of splicing in undifferentiated cells and further up-regulation of splicing during myogenic differentiation (lanes 2 and 3). The data obtained with both exon swaps are also consistent with the data of the exon deletions. A differentiation-dependent up-regulation of splicing was observed in the substituted and deleted variants, but in both cases some class-1 mRNA was already formed in undifferentiated cells. We also stably transfected the constructs containing the modifications of exon 25 in the C3H/10T1/2 fibroblast cell line. In none of the RNA samples prepared from these transfected cells some class-1 mRNA could be amplified (results not shown).

A strong acceptor site is required for muscle-specific splicing

In previous experiments [7], we demonstrated that weak 5′ splice donor sites and a long terminal intron are prerequisites for regulated muscle-specific splicing (Figure 1), but that there are no specific negative *cis*-acting sequences present in the optional terminal intron. In those experiments we did not analyse the sequence of the branchpoint and 3′ acceptor upstream of the SERCA2 optional exon 25, as it seemed to fit reasonably well with the mammalian consensus sequence. The SERCA2 branchpoint/acceptor sequence is GTG**A**T$(Y)_{21}$CAG (branchpoint A in bold) compared with the consensus sequence: CT(C/G)**A**C$(Y)_{20–40}$CAG [29,30]. In this study, we exchanged a 60 nt-long fragment spanning the branchpoint and 3′ splice site of SERCA2 for a 94 nt-long *Bsp*120I–*Eco*RI fragment containing the β-globin branchpoint and acceptor site of exon 3, a constitutively spliced exon (Figure 4A). The nucleotide sequence of the branchpoint/acceptor in this fragment is TTC**A**TG$(Y)_{16}$ACAG. As shown in Figure 4(B), this acceptor swap (AS1) did not interfere with the up-regulation of class-1 formation during differentiation, although some splicing could be detected in undifferentiated BC_3H1 cells.

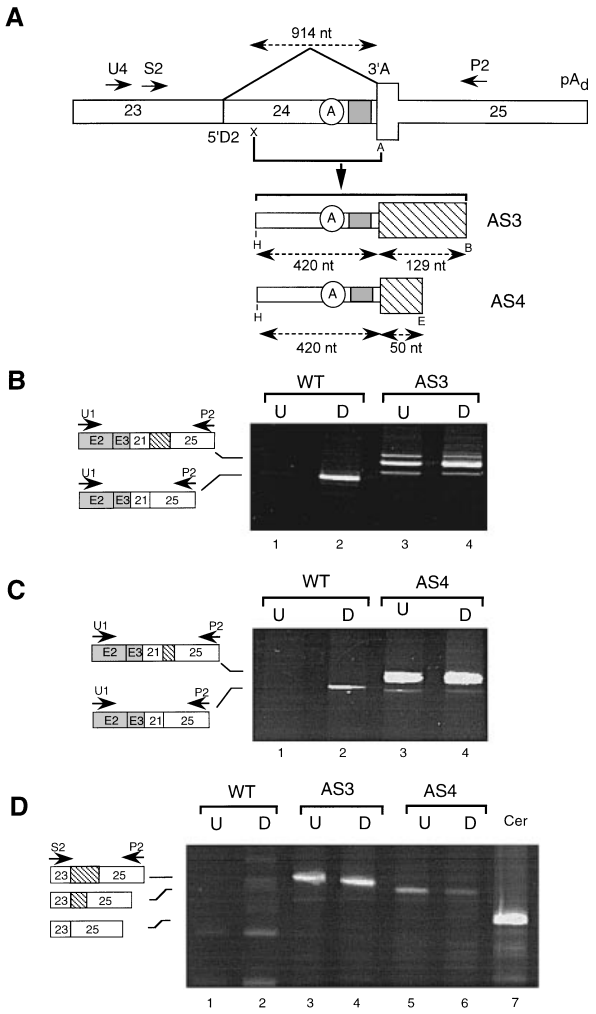

Figure 5 Effect of replacing the region upstream of the acceptor by a β-globin acceptor region on SERCA2 pre-RNA processing

(**A**) Schematic representation of the acceptor swaps 3 (AS3) and 4 (AS4). The enriched A indicates the branchpoint adenosine, and a shaded box represents a polypyrimidine tract. The acceptor of the muscle-specific exon was removed by eliminating a *Xho*I(X)–*Asp*718(A) fragment from the construct (Mut11). It was replaced by a *Hin*cII(H)–*Bg*lII(B) fragment and a *Hin*cII–*Eco*RI(E) fragment from the rabbit β-globin gene containing the acceptor region of exon 3. This resulted in AS3 and AS4 respectively. (**B**) and (**C**) The effect of both acceptor swaps on muscle-specific processing of pCMβSERCA2 transcripts is shown. RT-PCR on total RNA isolated from undifferentiated (U) and differentiated (D) BC_3H1 cells was performed using the U1 sense primer and the P2 antisense primer. (**D**) Effect of AS3 and AS4 on neuronal-specific splicing. The oligo(dT)-primed cDNA was subjected to a PCR using the U4 sense primer and the P2 antisense primer. From this PCR mixture, 1 μl was used for a second PCR with S2 as a sense primer. Lane 7 shows the amplification product starting from pig cerebellum RNA. Representations of the amplified fragments and the primers are shown on the left of the gels.

The SERCA2 acceptor was also exchanged for an acceptor of Igμ (AS2, Figure 4A) which has a branchpoint and acceptor sequence that contains a polypyrimidine tract interrupted by purines: $CTCACTA(Y)_2G(Y)_5A(Y)_3ACAG$. After transfection of this construct in BC_3H1 cells, no class-1 mRNA could be detected in undifferentiated or differentiated cells (Figure 4C). Class-2/3 mRNA was present in those cells, indicating normal transfection and expression of the minigene (results not shown). As it has been suggested that intervening purines may weaken the acceptor site, these data suggest that acceptor site strength is an important factor in splice regulation of SERCA2.

Sequences upstream of the muscle-specific acceptor site suppress the muscle type of splicing

We also exchanged a broader part (914 nt) of the 3′ acceptor region of exon 25 for two different fragments containing the 3′ acceptor region of exon 3 of the rabbit β-globin gene (AS3 and AS4; Figure 5A). The difference between the two acceptor swaps is that AS3 contained the first 129 nt of the β-globin exon 3 whereas this was reduced to 50 nt in AS4. Remarkably, AS3 and AS4 strongly induced muscle-type splicing in undifferentiated BC_3H1 cells (Figures 5B and 5C). For AS4, splicing was so strongly stimulated in undifferentiated muscle cells that no further up-regulation could be detected during myogenic differentiation (Figure 5C, lanes 3 and 4). We were also able to detect class-1 mRNA in C3H/10T1/2 fibroblasts after transfecting them with AS3 and AS4 (results not shown). These results strongly suggest that, in undifferentiated muscle cells as well as in non-muscle cells, sequences upstream of the acceptor region are involved in the suppression of muscle-type splicing of SERCA2.

Sequences upstream of exon 25 also interfere with the regulation of the neuronal type of splicing

From the previous results it was concluded that the region upstream of exon 25 is critically involved in the regulation of muscle-specific splicing. The same acceptor is used for the formation of the class-4 messenger which involves the selective removal of exon 24, a splicing process that is normally restricted to neuronal cells (Figure 1). We investigated whether the regulation of class 4 was also influenced in AS3 and AS4 (Figure 5A). Class-4 bands were amplified using the U4 and P2 primers in a first PCR and the S2–P2 combination in a second PCR as described previously. As a control we used RNA isolated from pig cerebellum (Figure 5D, lane 7). As shown in Figure 5(D), a class-4 band could be amplified in both undifferentiated and differentiated BC_3H1 cells for AS3 (lanes 3 and 4) as well as AS4 (lanes 5 and 6). Class-4 mRNA was not up-regulated during differentiation of BC_3H1 cells. The relative amounts of class-4 mRNA compared with class-1 mRNA cannot be deduced from these experiments. The exon swaps (ES1 and ES2) did not induce class-4 mRNA formation (results not shown). The only other condition so far in which class-4 messengers in non-neuronal cells could be detected was after changing the 5′D2 to the consensus sequence [7]. These results indicate that sequences surrounding the 3′ acceptor site upstream of exon 25 are not only involved in the formation of the muscle-specific class-1 mRNA but also in the regulation of the neuronal-specific class-4 mRNA formation.

DISCUSSION

A specific mechanism controls muscle-type alternative splicing of SERCA2 transcripts. It renders splicing of the alternative intron inefficient in non-muscle cells and up-regulates it during myogenic differentiation [6,8]. Different structural elements in the SERCA2 gene, such as a weak donor splice site and a long terminal intron, contribute to the low probability of splicing in non-muscle cells [7].

If the inability to splice in non-muscle cells was solely due to these structural elements, one could envisage a regulatory mechanism whereby a positive splice factor is expressed during muscle differentiation. This factor, by binding to a specific sequence in the SERCA2 transcript, would increase the probability of muscle-specific splicing. If so, removal or inactivation of the binding site for this factor would block the muscle-specific induction of splicing. Previously, the muscle-specific intron was screened for

the presence of specific *cis*-acting regions. Except for an intron length effect, no indications were found for the presence of an intronic splice enhancer [7]. In the present paper, we searched the muscle-specific exon 25 for positive *cis*-acting elements. However, deleting different parts of exon 25 or exchanging the entire exon for an unrelated sequence did not prevent the muscle-specific splice induction. These data invalidate the hypothesis that an exonic splice enhancer is required for muscle-type splicing. This conclusion is also in accordance with our previous observation that overexpression of an SR protein could not induce muscle-specific splicing [8].

In the present study, we provide evidence that the acceptor region of the optional intron and the preceding intronic part play a critical role in the regulation of splicing. Replacement of a 60 nt fragment containing the branchpoint and acceptor of the optional SERCA2 intron with a 82 nt fragment containing a branchpoint and acceptor of the Igμ gene precluded activation of splicing during myogenic differentiation. Interestingly, the Igμ gene branchpoint/acceptor region has a polypyrimidine tract that is relatively short and interrupted by several purines. It has been shown that a short and interrupted polypyrimidine tract reduces the efficiency of the acceptor site [31]. The effect of the Igμ substitution on regulated SERCA2 splicing therefore indicates that the branchpoint/acceptor site of the optional intron needs to have a minimal strength so that it can be used during muscle differentiation. Apart from a minimal strength, no specific sequence requirements seem to be imposed on the branchpoint/acceptor region. Indeed, replacement of the SERCA2 branchpoint/acceptor region with the branchpoint/acceptor region of rabbit β-globin did not interfere with regulated expression during differentiation. Both the SERCA2 and the rabbit β-globin branchpoint/acceptor region contain extended and uninterrupted polypyrimidine tracts indicating that they are strong processing sites.

An important question that follows from the previous observation is how a relatively strong acceptor site is excluded from the splice process in non-muscle cells. Our data indicate that a more extended region that encompasses the terminal part of the optional intron, the branchpoint/acceptor region and some parts of exon 25 is involved in the inhibition of splicing. For example, when the terminal part of the intron including the branchpoint/acceptor region was replaced with an intronic fragment from rabbit β-globin (AS3 and AS4), splicing was strongly induced in non-muscle cells. As selective replacement of the branchpoint/acceptor region had only a marginal effect on splicing in non-muscle cells (see above), this observation points to the intron region upstream of the branch point as a critical region for prevention of splicing in non-muscle cells. The first 677 nt of the intron sequence replaced in AS3 and AS4 had already been removed during previous intron-deletion experiments (intron deletion 2 [7]), but this deletion did not induce splicing in non-muscle cells. Therefore the non-overlapping part between intron deletion 2 and AS3/4 seems to be the critical region for suppression of the 3′ splice site. This region corresponds to the 237 nt that precede the branchpoint of the optional intron.

Not only was the regulation of class-1 mRNA formation disturbed by replacing the sequence surrounding the acceptor site, but also the control of the neuronal type of splicing. This indicates that the acceptor region is not only crucial for the regulation of the muscle-specific splicing but that it is suppressed in non-neuronal cells so that it prevents the neuronal types of splicing (i.e. the removal of optional exon 24) in these cells.

Regulated alternative splicing by selective repression of the 3′ splice site has been described in other splice systems, such as transformer [15–18] and β-tropomyosin [32–37] alternative splicing. At present two different theoretical models can explain the mechanism of negative 3′ splice site regulation. The first presumes the presence of a sequence around the 3′ splice site to which a negative *trans*-acting factor binds. The second involves the presence of mRNA secondary structures. The formation of hairpin loops, for instance, could make the splice site sterically inaccessible for spliceosome formation, as has been described for β-tropomyosin alternative processing [33–37]. Sequences surrounding the alternatively included muscle-specific exon 7 of β-tropomyosin were shown to be involved in the formation of a stable secondary structure.

On the basis of these models, two different mechanisms for the regulation of SERCA2 splicing can be formulated. (1) Splicing in non-muscle and non-neuronal cells is inhibited by the binding of a repressor to the 3′ part of the muscle-specific intron. During myogenic and neuronal differentiation, this factor is down-regulated and/or a positive *trans*-acting factor is produced that competes with the repressor for the same binding site. (2) Splicing in non-muscle and non-neuronal cells could be inhibited by the presence of a stable secondary structure spanning the 3′ splice site. This would make the 3′ splice site inaccessible for spliceosome formation. During myogenic or neuronal differentiation a splice factor destabilizing this secondary structure could be produced. It is interesting to note that some splice factors show RNA helicase activity as they contain the DEAD or DEAH motif characteristic of a large family of putative ATP-dependent RNA helicases [38,39].

The actual mechanism may be a combination of these two. Whatever the regulatory mechanism is, the region involved seems to be a large one situated around the 3′ splice site. What the exact contribution of the different parts within this region is and how the choice of the donor splice site is differentially made in muscle and neuronal cells have still to be investigated.

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